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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The methane-producing archaebacteria have a unique biochemistry and molecular biology. In this work, a collection of mutants in the thermophilic autotroph <u>Methanobacterium thermoautotrophicum</u> was obtained. Several of these were resistant to pyrimidine and purine analogs (e.g. 5-fluorouracil and 6-mercaptopurine). High molecular weight chromosomal DNA from some of the analog-resistant strains was used to transform wild type cells to resistance. A formate auxotroph was isolated, which represents a novel growth requirement. Characterization of this strain will reveal new aspects of one-carbon metabolism in methanogens. A complete genomic library of restriction fragments of the methanogen DNA was prepared in <u>Escherichia coli</u> plasmid pBR322. Transposon sequences will be introduced into these fragments, and linearized, transposon-containing plasmids used to transform the methanogen. This technique (additive transformation) followed by selective enrichment will yield mutants. The mutants and the ability to introduce DNA into recipient strains will provide the basis for genetic mapping and engineering of methanogens.					
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FINAL REPORT

Office of Naval Research Grant N00014-86-0222

"Development of a Gene Cloning System in Methanogens."

April 1986 through June 1988

The University of Oklahoma Department of Botany and Microbiology

Norman, Oklahoma

David P. Nagle, Jr., Michael J. McInerney, and David R. McCarthy.

I. Background and Objectives.

The methanogens, members of the group known as archaeobacteria, are biochemically novel and ecologically important. These organisms occupy the terminal position of anaerobic food chains, and permit the mineralization of large amounts of organic matter. Methane, the end product of their metabolism, contains a large percentage of the free energy of formation of the growth substrates and is a useful fuel substance. The cells survive by harnessing some of the energy available from the process of converting simple compounds (e.g. acetate, methanol, hydrogen and carbon dioxide) to methane.

Modern techniques of microbiology permit the facile culture and manipulation of these strict anaerobes. Techniques of molecular biology in eubacterial host-vector systems permit the isolation of genes, sequencing, the recognition of structurally important regions, and study of regulation. A key to progress is to reintroduce these genes, altered genes, or new genes back into the methanogen system.

The objectives of this work follow.

- A. To develop a cloning system for methanogens which includes the following.
 - 1. Isolation of mutants whose phenotypes permit direct selection.
 - 2. Mechanism for genetic transfer among strains.
 - 3. Construction of plasmid vectors for genetic exchange.
- B. To characterize the physiological properties of mutants.
- C. In the long term, to understand aspects of cellular metabolism and mechanisms for adaptation to growth on different substrates, and to set the stage for engineering of methanogen strains with altered properties, such as the ability to use new growth substrates or electron donors.

II. Experimental Approach.

The primary organism under investigation is the autotrophic, thermophilic methanogen Methanobacterium thermoautotrophicum strain Marburg.



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Mutants in this strain were selected for resistance to a variety of purine and pyrimidine analogues and to kanamycin. Resistant strains were obtained from single colony isolates that arose spontaneously or after chemical mutagenesis. The characterization of mutants for their ability to activate purine and pyrimidine analogues and to transport bases was carried out as described in the appendix (Nagle et al., 1987). A formate auxotroph (strain RT103) was obtained after hydroxylamine mutagenesis and two cycles of enrichment by outgrowth in mineral medium in the presence of bacitracin. Characterization of this mutant was done in anoxic cell-free extract prepared with the French pressure cell. Spectrophotometric formate dehydrogenase assays were carried out at 60° in anaerobic cuvettes with various electron acceptors. Specific activities were expressed as $\mu\text{mol electron acceptor reduced/min} \times \text{mg protein}$. The transformation system in which resistance to pyrimidine and purine analogues was transferred via chromosomal DNA from resistant strains is described in the appendix (Worrell et al., 1988). A library of *M. thermoautotrophicum* PstI fragments of chromosomal DNA has been prepared in plasmid pBR322 and maintained in *E. coli*.

III. Results.

M. thermoautotrophicum strain Marburg was chosen as the primary experimental organism for this work for several reasons. It takes up a variety of substrates (e.g. Nagle et al., 1987), although it is an autotroph. Numerous mutants have been obtained, and the strain contains a well-characterized plasmid. The organism plates at high efficiency. Much of the enzyme and coenzyme biochemistry has been worked out in *M. thermoautotrophicum* strains Marburg or H, so that this group is the best understood among the methanogens. However, the major reason for selecting *M. thermoautotrophicum* strain Marburg instead of the well-characterized *Methanococcus voltae* was our success in obtaining transformation of wild-type cells with DNA from mutants that were resistant to antimetabolites (Worrell et al., 1988). These mutants exhibited elevated resistance to nucleic acid base analogues. Unlike the wild-type, which in each case is completely prevented from growth at drug concentrations of about 0.1 mg/ml, the resistant strains are capable of growth at concentrations of 1 mg/ml or greater. A list of these drug-resistant strains is presented in Table 1. The spontaneous mutation frequencies for some of the phenotypes shown in the table were as follows: for 8-azahypoxanthine, 6-methylmercaptapurine riboside, and 5-fluorouridine, $1 - 5 \times 10^{-6}$; for 5-fluorouracil and 5-fluorodeoxyuridine, 5.3×10^{-8} and 3.5×10^{-9} , respectively; and for 6-mercapto purine at 0.3 mg/ml, 1.5×10^{-6} , and at 1 mg/ml, $<3 \times 10^{-6}$ (Nagle et al., 1987; Worrell et al., 1988; Worrell et al., unpublished results). The fluorouracil-resistant strain RTAE-1 was deficient in the enzyme uracil phosphoribosyltransferase, which activates the base to the nucleotide. This deficiency was sufficient to explain resistant phenotype, since the mutant could not produce the toxic nucleotide. The mechanism of resistance in the purine analogue-resistant strains is not known. However, we have determined that extracts of the wild type strain contain adenine phosphoribosyltransferase activity (unpublished data), which could activate the base analogues to inhibitory nucleotides. The phenotypes 5-fluorouracil-resistance and 6-mercaptapurine resistance have been transferred into wild-type cells with chromosomal DNA from the resistant

strains RTAE-1 and RTVW-1 (Worrell et al., 1988, appendix; Worrell et al., unpublished results). The key to this transformation system appears to be in the use of Gelrite gellan gum as the solidifying agent, and in performing transformation during growth under non-selective conditions on the surface of the plates. We have not obtained transformation under a variety of conditions in liquid culture nor on the surface of agar plates. Those variables which permit cells to become transformed in our system remain unknown.

A formate auxotroph was obtained in experiments in which strain RT-100 was treated with ethylmethanesulfonate or hydroxylamine at several different concentrations each. Mutagenesis was followed by two rounds of enrichment for auxotrophs in mineral medium containing bacitracin. Bacitracin apparently kills actively growing wild-type cells while mutants that are starved for essential nutrients survive the exposure to the drug. Each cycle was followed by outgrowth in nutrient-supplemented medium (auxotroph medium). Out of 96 colonies from hydroxylamine mutagenesis which grew on auxotroph medium, 23 would not grow on mineral medium. By eliminating nutrient pools one at a time from the auxotroph medium, it was found that ten of the 23 auxotrophic strains required formate for growth. Nutritional studies on strain RT-103 (Table 1) showed that 200 mg per liter of sodium formate was required for normal growth rates and cell yields. Preliminary results showed that [^{14}C]-formate was incorporated into soluble, lipid, RNA, DNA, and protein pools in cells of both the formate auxotroph and the wild type (Tanner et al., unpublished results). Cell-free extracts of strains RT-100 (kanamycin-resistant wild type) and strain RT-103 were screened for the presence of formate dehydrogenase activity. This enzyme had not been described previously in M. thermoautotrophicum. Although no activity was detected with the artificial electron acceptor benzyl viologen, extracts of the wild type carried out formate-dependent reduction of both NAD and NADP. Specific activities for these electron acceptors were as follows: in strain RT-100, 3.8 and 5.7; in strain RT-103, -0.6 and 2.8 (Tanner, unpublished results). The negative value for strain RT-103 is due to suppression of the formate-independent background activity in undialyzed extracts. Clearly these two strains can be distinguished biochemically on the basis of their ability to reduce NAD with formate.

IV. Prospects.

The basis for significant progress in understanding the genetics and physiology of M. thermoautotrophicum has been established. The collection of resistant mutants will permit the determination of the pathways of nucleic acid base metabolism in this organism. The several markers can be used to attempt to establish a linkage map by use of the chromosomal DNA-dependent transformation system. The discovery of a formate auxotroph, which is a unique requirement, opens a new view of one-carbon metabolism in the methanogens. The results of formate labeling and the demonstration of formate dehydrogenase activity suggested a novel method of one carbon activation which will be pursued and described. New molecular techniques can be applied to our genetic system. The technique of additive transformation will be utilized to prepare transposon insertions in methanogen restriction fragments maintained in our Escherichia coli plasmid pBR322 library. The transposon miniTn10 can

insert randomly into DNA sequences. A mixture of chimeric plasmids containing Tn10 insertions will be linearized and used to transform M. thermoautotrophicum. If the insert-containing DNA recombines with the chromosome, insertion mutants will result. Auxotrophs can be selectively enriched, and base analogue-resistant variants isolated directly. This technique promises to be an efficient method of mutagenizing chromosomal genes. In addition, the defective alleles can be isolated by cloning the mutated DNA into E. coli, and then used to isolate the wild-type sequences.

Together with the body of biochemical, structural, sequence, and regulatory information which is being produced by other laboratories, our system will add to basic knowledge of methanogens. The ability to reintroduce genes will permit the engineering of strains with the ability to utilize new substrates or electron donors, or with other useful properties.

V. Publications.

Nagle, D.P., Jr., R. Teal, and A. Eisenbraun. 1987. 5-Fluorouracil-resistant strain of Methanobacterium thermoautotrophicum. J. Bacteriol. 169: 4119-4123.

Worrell, V.E., D.P. Nagle, Jr., D. McCarthy, and A. Eisenbraun. 1988. Genetic transformation system in the archaeobacterium Methanobacterium thermoautotrophicum Marburg. J. Bacteriol. 170: 653-656.

Manuscripts in preparation include: the isolation and characterization of the formate auxotroph, fluorouracil derivatives as inhibitors of archaeobacteria, characterization of analogue resistant strains, and optimization of the genetic transformation system. We were invited to present a symposium talk entitled "Development of genetic systems for methanogenic archaeobacteria" at the Annual Meeting of the Society for Industrial Microbiology in August, 1988, and a companion manuscript will be submitted to Developments in Industrial Microbiology.

VI. Abstracts and Presentations.

Nagle, D.P. Jr., V.E. Worrell, C.W. Jones, and R. Teal. 1987. The folic acid content of archaeobacteria and response to antifolate compounds. Abstr. Annu. Meeting Amer. Soc. Microbiol. I 130, p. 194.

Tanner, R.S., and D.P. Nagle, Jr. 1987. Effect of sulfanilamide and folate analogues on the growth of Methanomicrobium mobile. Abstr. Ann Meeting Amer. Soc. Microbiol. I 34, p. 178.

Nagle, D.P., Jr., V.E. Worrell, D. McCarthy, and A. Eisenbraun. 1987. Natural genetic transofrmation system for the archaeobacterium Methanobacterium thermoautotrophicum. Abstr. 2nd Mid-America Molecular Biology Colloquium, p. 31.

Nagle, D.P., Jr., V.E. Worrell, A. Eisenbraun, and D. McCarthy. 1987. DNA-dependent transformation system for Methanobacterium thermoautotrophicum.

Gordon Research Conference, "Molecular aspects of methanogenesis", New Hampton, New Hampshire, 29 June-3 July.

Tanner, R.S., and D.P. Nagle, Jr. 1988. Mutagenesis and mutant selection of Methanobacterium thermoautotrophicum. Abstr. Annu. Meeting Amer. Soc. Microbiol. I 10.

Nagle, D.P., Jr. "Genetic engineering and the energy crisis." Invited talk presented to the Tinker Society of Professional Engineers and Scientists, Tinker Air Force Base, 26 November, 1986.

Nagle, D.P., Jr. 1988. Development of genetic systems for methanogenic archaeobacteria. Abstr. Annu. Meeting Soc. Indust. Microbiol., in press.

VII. Awards and Honors.

M.J. McInerney, University of Oklahoma Associates Distinguished Lectureship, 1986-1987.

D.R. McCarthy, Junior Faculty Summer Research Fellowship, University of Oklahoma, 1987.

D.P. Nagle, Jr., Junior Faculty Summer Research Fellowship, University of Oklahoma, 1987.

VIII. Personnel supported.

Dr. Ralph S. Tanner was supported in part by this grant as a Visiting Assistant Professor in the Department of Botany and Microbiology.

Doris M. Kupfer was supported in part as a laboratory technician.

Ms. Veronica E. Worrell, a third year graduate student, was supported for the grant period (Hispanic female, USA).

IX. Appendix.

Table 1. Mutants of Methanobacterium thermoautotrophicum isolated in this laboratory.

Report distribution list.

Manuscripts by Worrell et al., and Nagle et al.

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Genetic Transformation System in the Archaeobacterium *Methanobacterium thermoautotrophicum* Marburg

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A wild-type strain of *Methanobacterium thermoautotrophicum* Marburg was transformed by DNA from strains resistant to 5-fluorouracil. Recipient cells were grown without selection on gellan gum (GELRITE) plates with DNA. Drug-resistant cells were recovered by replica plating the resulting colonies onto drug plates. Transformation required high-molecular-weight DNA with appropriate markers and was not observed on agar or in liquid media under a variety of conditions.

A significant amount of molecular information has been uncovered about methanogenic archaeobacteria with the use of methanogen DNAs that have been cloned into eubacterial hosts (7, 9). A method for performing genetic experiments in methanogens is necessary so that the cloned genes can be used as starting points for meaningful physiological studies. Techniques for mutant selection and methods for introducing methanogen genes into their original backgrounds are the minimal requirements for a genetic system. Some progress has been made in this direction for several species of archaeobacteria. Several drug-resistant and auxotrophic mutants have been reported in various methanogen species (3, 8, 10, 11, 18, 20, 22). Among the halophilic archaeobacteria, transfer mechanisms based on conjugation and transfection with DNA from a lytic phage have been demonstrated (6, 17). Genetic transformation of the mesophile *Methanococcus voltae* PS in liquid culture was reported recently (3).

We present evidence for a natural transformation system in the thermophilic methanogen *Methanobacterium thermoautotrophicum* Marburg. A marker for 5-fluorouracil (FU) resistance was transformed into the wild-type strain via DNAs from drug-resistant mutants.

MATERIALS AND METHODS

Strains and media. Table 1 lists the strains used in this study. *Halobacterium volcanii* was grown in medium 97 at 37°C (5). *Escherichia coli* was grown in LB broth at 37°C. *M. thermoautotrophicum* strains were grown on mineral medium 2 as described previously (1, 24). Mineral medium plates contained 0.8% GELRITE gellan gum (Kelco Division of Merck & Co., Inc., San Diego, Calif.) plus a cation mixture composed of 1 g each of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ per liter. The amount of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ was reduced 10-fold to a final concentration of 0.006%. Mineral medium agar plates contained 2% Noble agar (Difco Laboratories, Detroit, Mich.) with or without the cation mixture. All plating operations, including preparation of plates after sterilization of anoxic medium, were done in an anaerobic chamber under an atmosphere of 95% N_2 and 5% H_2 (2). Additions were made by spreading liquid supplements on the solidified medium with a bent glass rod or a sterile plastic loop. Plates were incubated in pressure vessels (modified from references 1 and 2) under an atmosphere of H_2 - CO_2 - H_2S (80:19:1 [vol/vol]) at 200 to 300 kPa for 2 to 4 days at 60°C. To score colonies, the gas phase was exchanged with

nitrogen, and the vessels were brought into the anaerobic chamber.

DNA purification. *M. thermoautotrophicum* cells were suspended in SE solution (0.15 M NaCl, 0.01 M sodium EDTA, pH 8.0; 1 g [wet wt] per 7 ml of buffer) and passed once through a French pressure cell at 40 or 130 MPa. This lysis technique produced a broad size distribution of DNA fragments (0.5 to 20 kilobase pairs). Lysates that were prepared as described by Meile et al. (16) yielded uniformly high-molecular-weight DNA fragments. *H. volcanii* cells were lysed osmotically by suspension in SE solution. *E. coli* (in SE solution) was frozen at -20°C, thawed, and treated with lysozyme (1 mg/ml, 5 min, 4°C). The DNA was purified by the Marmur technique after lysis (12). Purified DNAs were stored at -20°C in 0.1 × SSC buffer (0.015 M NaCl, 1.5 mM trisodium citrate, pH 7.0). The size of *M. thermoautotrophicum* DNA was determined by agarose gel electrophoresis with *Hind*III-cut phage lambda DNA as a molecular weight standard. Low-molecular-weight DNAs (size, <0.5 kilobase pairs) were produced by shearing high-molecular-weight samples three times with a French pressure cell.

Genetic transformation. DNA samples (2 ng to 100 µg) were placed at the centers of plates, and inocula (0.05 to 0.2 ml) containing 100 to 200 CFU were spread over the DNA-containing regions with sterile, disposable plastic loops. In some experiments, enough cells were added to the DNA plates to cause confluent growth; however, this modification had no effect on the results. Controls (plates lacking cells or DNA) were run with every experiment. After 2 to 4 days of incubation at 60°C, the resulting colonies were replica plated onto selective media. Replica plating was done with wooden applicator sticks (2-mm diameter) held perpendicular to the plates. Each inoculum was replica plated by impressing the end of an applicator stick onto the gellan gum surface so as to maintain the organization of the original colony. Any sectorized distribution of drug-resistant cells in a colony was preserved on the selective plates. In several of the early experiments, a random sample of transformants was tested for the ability to produce methane at 60°C to ensure that they did not represent contaminating organisms.

Transformation in liquid medium was attempted as follows. Cultures containing 1.1×10^7 cells per ml were incubated with DNA (0.9 µg/ml from strain RTAE-1 or 51 µg/ml from the wild type) for 6 h with gentle shaking or for 48 to 72 h with or without shaking. A sample of 1×10^7 to 2×10^7 cells from each of these conditions was plated on FU. In some experiments, DNA was added with polyethylene

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TABLE 1. Strains

Strain	Proposed genotype ^a	Phenotype	Source or reference
<i>M. thermoautotrophicum</i> Marburg			
DSM 2133	<i>fus</i> ⁺	FU ⁺	H. Hippe
RTAE-1	<i>fus</i> -1	FU ⁻	DSM 2133; 18
RTVW-1	<i>fus</i> -1 <i>mps</i> -1 ⁻	FU ⁺ MP ⁻	RTAE-1; this work
<i>E. coli</i> ATCC 8739 ^b			K. Kealy
<i>H. volcanii</i> ATCC 29605			ATCC

^a The *fus* locus encodes sensitivity or resistance to FU. The *mps*-1 allele encodes resistance to 6-mercaptopurine.

^b ATCC, American Type Culture Collection, DSM, Deutsche Sammlung von Mikroorganismen.

glycol (PEG) 6000 (final concentration, 20% [wt/vol]) or PEG 6000 and the cation mixture that was used in the gellan gum plates (1 g each of MgSO₄ · 7H₂O and CaCl₂ · 2H₂O per liter). No attempt was made to convert the culture into protoplasts when PEG was included in the medium (14). The presence of transformants was measured by plating the cells on selective plates.

RESULTS AND DISCUSSION

The drug resistance phenotype FU⁺ was used to assay for transformation of *M. thermoautotrophicum*. This phenotype was attractive, because it appeared at low frequencies by spontaneous mutation ($<1.6 \times 10^{-8}$). The FU⁺ strain RTAE-1 arose as a spontaneous mutant with a defect in uracil phosphoribosyltransferase (18). Strain RTVW-1 was selected from a culture of strain RTAE-1 which spontaneously grew in the presence of 6-mercaptopurine (500 µg/ml; Table 1).

Genetic transformation of *M. thermoautotrophicum*. We tried several protocols for transformation of *M. thermoautotrophicum* cells with DNA from FU⁺ strains RTAE-1 and RTVW-1. Liquid cultures were exposed to DNA in the presence of 20% PEG and cations or in the absence of these supplements. PEG in the liquid inoculum protected the plated cells from inhibition by FU, but both methods failed to yield drug-resistant cells at frequencies greater than those of spontaneous mutation. We then reasoned that competence could be associated with a particular physiological state that might not be accessible to most cells in liquid cultures (19, 23). Therefore, we tried transformation experiments on plates on the assumption that cells might pass through a spectrum of physiological states which might include competence (13, 19). In our first experiments, strain RTAE-1 DNA was placed on the surfaces of gellan gum plates, and wild-type cells were allowed to grow into confluent patches over the DNA region. We tested several hundred regions of these confluent lawns and found FU⁺ cells in 3% of the samples when the cells were exposed to FU⁺ DNA but not wild-type DNA. Drug-resistant cells were not recovered from plates that lacked DNA (data not shown). FU⁺ cells were recovered from the confluent patches from the DNA plates only if transformation was allowed to proceed in the absence of selection. As expected, it was important to allow nonselective growth in the presence of FU⁺ DNA before challenging the cells with the drug to allow time for the development of competence, transformation, and expression of the drug resistance allele.

The transformation experiments were repeated with smaller inocula so that single colonies formed on the DNA plates. The number of colonies which contained a subset of FU⁺ cells was dependent on the amount of FU⁺ DNA added (Fig. 1). Transformation was expressed as the percentage of colonies that contained drug-resistant cells. No evidence of transformation was seen in the absence of DNA (825 colonies tested). With as little as 2 ng of DNA from strain RTAE-1, FU⁺ cells were found in 1.1% of colonies; the frequency of resistant cells reached a plateau value at 10 ng of DNA per plate. When colonies were replica plated so as to preserve the original distribution of sibling cells, we found that transformants were almost always at the edges of the colonies. We never recovered colonies that contained a majority of drug-resistant cells. This result implied that transformation rarely if ever occurred early in the development of a colony.

The appearance of FU⁺ cells was specific for FU⁺ DNA from *M. thermoautotrophicum* (Table 2). DNAs from the archaeobacterium *H. volcanii* or the eubacterium *E. coli* did not produce drug-resistant *M. thermoautotrophicum* cells. Only one colony containing FU⁺ cells appeared in 760 that were tested when wild-type (FU⁻) strain Marburg was the

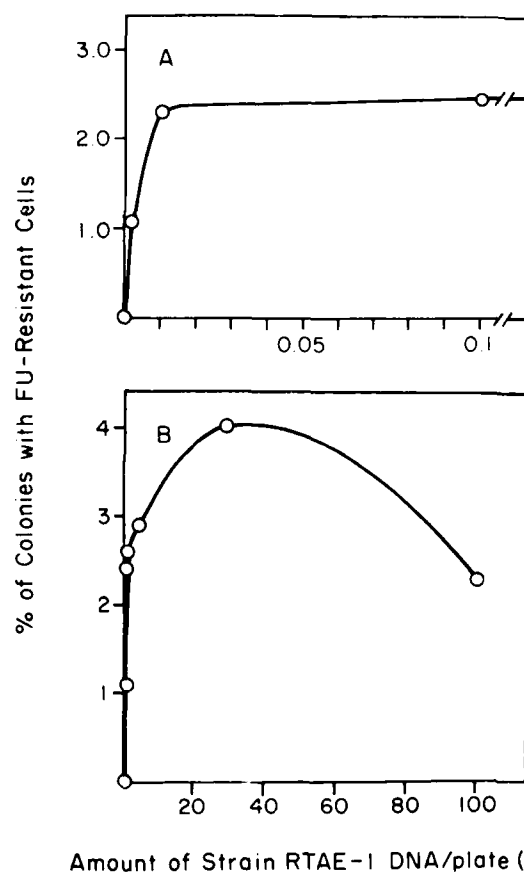


FIG. 1. Transformation of wild-type *M. thermoautotrophicum* with strain RTAE-1 DNA. DNA from FU⁺ strain RTAE-1 was placed at the centers of mineral medium plates containing GEL-RITE gellan gum. The region with DNA was spread with cells of *M. thermoautotrophicum* Marburg. At least 125 colonies were tested at each concentration of DNA. (A) Transformation data for low concentrations of DNA on an expanded scale. (B) Data for the complete range of DNA concentrations tested.

TABLE 2. Effects of media and DNA source on transformation

Medium and source of DNA	No. of colonies tested ^a	No. of colonies with EU ⁺ cells
GELRITE		
RTAE-1, RTVW-1 (EU ⁺)	1,062	14
RTAE-1 (sheared)	154	0
Marburg (wild type)	760	1
<i>H. volcanii</i>	99	0
<i>E. coli</i>	216	0
None	825	0
Agar		
RTAE-1	395	0
Marburg	378	0
None	389	0

^a Colonies were grown on minimal plates under nonselective conditions for 2 to 4 days and then replica plated onto minimal and EU (100 µg/ml)-containing plates. EU⁺ was confirmed by replating.

source of transforming DNA. This isolate probably arose by spontaneous mutation. The dependence of transformation on an appropriately marked *M. thermoautotrophicum* DNA implies that drug-resistant cells arose as a result of recombination and not from the induction of SOS or error-prone repair systems. If exposure of the cells to DNA induced mutation, we would expect DNA from any source to induce drug-resistant pseudotransformants.

Efficient transformation was dependent on high-molecular-weight DNA. The DNA listed in line 1 of Table 2 had an average size of about 20 kilobase pairs; fragments of about 0.5 kilobase pairs did not support transformation (Table 2, line 2).

Gellan gum-dependent transformation. GELRITE gellan gum (15) was used in our initial experiments because of its ability to absorb relatively large volumes of water from supplements and because it remained dry after incubation at elevated temperatures. We attempted transformation experiments on the surface of Noble agar but obtained no transformants even at high amounts of DNA per plate (Table 2). The magnesium-calcium cation mixture was added to the agar in some plates to see whether high concentrations of cations could enhance transformation, but this modification inhibited growth of the cells. However, transformation was obtained in experiments with different lots of gellan gum and several preparations of marked DNA made by different methods and performed by different investigators. The reasons for our success with GELRITE gellan gum and not with Noble agar are unclear. It was not likely that the Mg-Ca cation mixture in gellan gum medium was solely responsible for the transformation, since it could not induce transformation in liquid or on agar plates. The only other variable was the use of gellan gum versus agar to solidify the DNA plates. Therefore, it is possible that the structure of the gellan gum matrix is involved in transformation. Gellan gum is a linear polymer of tetrasaccharide that contains glucuronic acid (21). The stiff helical structure of the polymer in solution (4) and the regular alignment of negative charges in the solidified gel might influence the conformation of the DNA or the cell wall to enhance competence.

The transformation system for the pseudomurein-containing *M. thermoautotrophicum* (1) on gellan gum plates is simple, reproducible, and specific for high-molecular-weight DNA with the appropriate marker. No special techniques, such as protoplast formation, heat shock, or starvation, are required. The results suggest that, with auxotrophic mutants and vectors already available, progress on the genetic un-

derstanding of pseudomurein-containing methanogens will accelerate.

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5-Fluorouracil-Resistant Strain of *Methanobacterium thermoautotrophicum*

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Growth of *Methanobacterium thermoautotrophicum* Marburg is inhibited by the pyrimidine, 5-fluorouracil (FU). It was shown previously that methanogenesis is not inhibited to the same extent as growth. A spontaneously occurring FU-resistant strain (RTAE-1) was isolated from a culture of strain Marburg. The growth of both strains was inhibited by 5-fluorodeoxyuridine but not 5-fluorocytosine, and the wild type was more susceptible to inhibition by 5-azauracil and 6-azauracil than was strain RTAE-1. The cellular targets for the pyrimidine analogs are not known. When the accumulation of ^{14}C -labeled uracil or FU by the two strains was compared, the wild type took up 15-fold more radiolabel per cell than did the FU-resistant strain. In the wild type, radiolabel from uracil was incorporated into the soluble pool, RNA, and DNA. The metabolism of uracil appeared to involve a uracil phosphoribosyltransferase activity. Strain Marburg extracts contained this enzyme, whereas FU-resistant strain RTAE-1 extracts had less than 1/10 as much activity. Although it is possible that a change in permeability to the compounds plays a role in the stable resistance of strain RTAE-1, the fact that it lacks the ability to metabolize pyrimidines to nucleotides is sufficient to account for its phenotype.

The description of methane-producing archaeobacteria is a collection of biochemical variations on familiar themes. Many of the themes have to do with the pathways of methanogenesis and autotrophy and with the cofactors and structural molecules used by the cell (1, 15, 16, 33). Some of the other aspects of metabolism, for example, many of the anabolic reactions and their regulation, repair functions, and salvage mechanisms remain to be clarified. As part of an ongoing study of the coenzymes of methanogens, we chose to work with *Methanobacterium thermoautotrophicum* Marburg, which is an autotroph but appears to take up a number of substrates (7, 8). Enzyme preparations from this thermophile tend to be stable in vitro. Some mutants have been obtained (17). For further genetic work, the plasmid pME2001 and vectors based upon it may be very useful (21, 22).

We previously determined that 5-fluorouracil (FU), a uracil analog, is a potent inhibitor of the growth of *M. thermoautotrophicum* (31). Methanogenesis is not inhibited to the same extent as growth. Here we describe the characterization of a spontaneous isolate which was capable of growth in high concentrations of FU. The characterization of this isolate and its parent provided evidence of the existence of a pyrimidine salvage pathway in methanogenic archaeobacteria.

(A preliminary report of some of the results has been presented [D. P. Nagle, Jr., A. Eisenbraun, and R. Teal, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, 150, p. 173].)

MATERIALS AND METHODS

Sources of chemicals. Bulk chemicals were reagent grade. Uracil, uridine, UMP, FU, 5-fluorodeoxyuridine, and 5-fluorocytosine were from Sigma Chemical Co., St. Louis, Mo.; Omnifluor was from New England Nuclear Corp., Boston, Mass.; $[2\text{-}^{14}\text{C}]\text{FU}$ (58 mCi/mmol) and $[2\text{-}^{14}\text{C}]\text{uracil}$ (53.2 mCi/mmol) were from ICN Radiochemicals, Irvine, Calif.; and phosphoribosylpyrophosphate (PRPP) and cys-

teine were from Calbiochem-Behring, San Diego, Calif. Alkaline phosphatase from calf intestine (EC 3.1.3.1) was from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

Media and growth conditions. *Escherichia coli* CSH36 (*lacI*) (23) from the department collection (K. Kealy, curator) was grown at 37°C in medium containing, per liter, 5.6 g of KH_2PO_4 , 28.9 g of K_2HPO_4 , 10 g of yeast extract, 10 mg of thiamine, and 10 g of glucose. *M. thermoautotrophicum* Marburg (DSM2133; obtained from H. Hippe) was grown at 63°C in medium 2 (1) with trace vitamins omitted as described previously (31). Anoxic, sterile solutions of drugs or isotopic tracers were added to 5- or 20-ml cultures. Growth was quantitated by absorbance and converted to number of cells by using a standard curve (31). Large amounts of methanogen cell mass needed for enzyme studies were obtained by using a 3-liter Microferm fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) maintained at 60 to 63°C and sparged with 1 liter of an 80% H_2 -20% CO_2 gas mixture per min.

Isolation of FU-resistant strain. A culture of *M. thermoautotrophicum* Marburg containing 100 μg of FU per ml grew after a prolonged lag period. This culture was continually passaged numerous times in the medium described above with 100 μg of FU per ml. The culture was diluted and plated on medium 2 with 10-fold less Na_2S , 1.5% (wt/vol) Noble agar (Difco Laboratories, Detroit, Mich.), and 1 mg of FU per ml. The plates were incubated under 200 kPa of an $\text{H}_2\text{-CO}_2\text{-H}_2\text{S}$ (80:19:1) gas phase for 2 days at 63°C in stainless steel pressure vessels modified from those described previously (1). A single, well-isolated colony was picked and designated strain RTAE-1.

Uptake of ^{14}C -labeled compounds. For uptake of ^{14}C -labeled compounds, a modification of a previous method was used (2). Membrane filters (pore size 0.45 μm ; diameter, 25 mm; Millipore Corp., Bedford, Mass.) were presoaked in buffer (medium 2 without resazurin or cysteine-sulfide reducing solution) containing 1 mg of uracil per ml. At the times indicated, duplicate 0.2-ml samples were anoxically removed from cultures and rapidly vacuum filtered. After

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two 1.0-ml washes with the buffer described above without uracil, the radioactivity trapped on the filters was determined. Each filter was placed in 2.7 ml of Omnifluor scintillation fluid (2.07 g of Omnifluor, 369 ml of Triton X-100, 631 ml of toluene) and 0.3 ml of water in a vial, and the radioactivity was counted in a Beckman LS-100C counter (Beckman Instruments, Inc., Fullerton, Calif.). Values were corrected for radiolabel that bound to filters in the absence of cells. Corrected values for uptake by strain RTAE-1 were at least three times this background, and values for the wild-type strain were correspondingly higher. Quench correction was not required, as determined by the channels-ratio method. In experiments with [2-¹⁴C]FU, the radiolabeled compound was added to growing cultures to a final concentration of 41 μ M and 1 μ Ci/ml. In experiments with [2-¹⁴C]uracil, the labeled compound was at a final concentration of 48 μ M and 0.7 μ Ci/ml. In the uracil labeling studies, the entire culture was needed for filtration of samples; therefore, growth was monitored by diluting parallel cultures for absorbance measurements. These cultures were prepared and handled identically to those with label except that [¹⁴C]uracil (but not [¹²C]uracil) was omitted.

For determination of the fate of radiolabeled uracil, cultures (20 ml) with 4.1 μ M [¹⁴C]uracil at 0.22 μ Ci/ml were grown as described above, centrifuged ($10,000 \times g$ for 10 min at 4°C), suspended in medium 2 without cysteine-sulfide reducing solution, and recentrifuged. The cell pellet was then fractionated by using a modification of published procedures (32). The cell pellet (40 mg [wet weight]) was suspended in 1 ml of 5% trichloroacetic acid (TCA) and incubated on ice for 30 min. The suspension was then centrifuged ($15,600 \times g$ for 5 min at 4°C). The supernatant was designated the cold TCA-soluble fraction, and the pellet was resuspended in 1 ml of 5% TCA and incubated in a boiling water bath for 30 min. After centrifugation (as above), the supernatant was designated the hot TCA-soluble fraction. The pellet was suspended in 1 ml of 0.3 M NaOH and designated the TCA-insoluble fraction. The radioactivity in these fractions was determined in triplicate as described above.

Assay of uracil phosphoribosyltransferase. Cells of *E. coli*, *M. thermoautotrophicum* Marburg, and *M. thermoautotrophicum* RTAE-1 were harvested by centrifugation at $10,000 \times g$ for 10 min at 4°C and washed by suspension in 100 mM potassium phosphate (pH 7.2) followed by recentrifugation. Cell pellets were stored at -20°C. To prepare extracts, cells were suspended in buffer (50 mM Tris hydrochloride [pH 7.4], 10 mM MgCl₂, and 7 mM 2-mercaptoethanol for *E. coli*; 50 mM Tris hydrochloride [pH 7.6], 1 mM EDTA, and 5 mM 2-mercaptoethanol for the methanogens). The cells were broken by passage through a French pressure cell two times at 138 MPa. After centrifugation ($31,000 \times g$ for 30 min at 4°C), crude extracts were stored at -20°C. Protein concentrations were determined by the Coomassie blue dye binding method with bovine serum albumin as the standard (4). Assay mixtures for determining uracil phosphoribosyltransferase activity (100 or 200 μ l) contained 50 mM Tris hydrochloride [pH 7.6], 5 mM MgCl₂, 200 μ M [¹⁴C]uracil (1 μ Ci/ml), 1 mM PRPP_i, and 3 to 5 mg of protein per ml. The incubation temperatures were 37°C for *E. coli* and 55°C for *M. thermoautotrophicum* (occasionally 63°C was used for the latter organism, with little difference in results). In some experiments, additional 10- μ l samples of PRPP_i (final concentration, 1 mM) were added after 1 h of incubation. Assay mixtures were spotted on cellulose thin-layer chromatography plates (6064; Eastman Kodak Co., Rochester, N.Y.). To

TABLE 1. Effects of pyrimidine derivatives on growth of FU-resistant *M. thermoautotrophicum* RTAE-1

Addition ^a	Growth (mean [SE]) ^b	% of control
None	0.61 (.08)	100
FU	0.53 (.06)	87
5-Fluorodeoxyuridine	0.06 (.02)	10
5-Fluorocytosine	0.57 (.08)	95

^a Compounds were added at 100 μ g/ml. Tubes were incubated for 49 h at 63°C.

^b Increase in A_{660} . Each value is the mean of at least three individual cultures (\pm standard error of the mean).

detect the nucleotide or nucleoside products, the plates were developed in one of three solvent systems: I, saturated (NH₄)₂SO₄-1 M sodium acetate-isopropanol (80:18:2) (10); II, butanol-water (86:14) (10); III, isobutyrate-0.5 N NH₄OH-*n*-butanol (60:36:4) (modified from solvent 3 of Grippo et al. [10]). Known standards were detected visually by UV quenching. The R_f values in these systems, respectively, were as follows: uracil, 0.57, 0.43, and 0.63; uridine, 0.68, 0.30, and 0.63; UMP, 0.72, 0.0, and 0.36. [¹⁴C]uracil was shown to be greater than 97% radiochemically pure in system I. Samples of assay mixtures were cochromatographed with 20 nmol of uracil and UMP. The lanes were cut into small segments which were placed in scintillation vials. The radioactivity in the vials was determined by scintillation counting as described above. The counting efficiency (91.1%) was not affected by the presence of the thin-layer sheet or coating. Some samples were treated with alkaline phosphatase by incubation of reaction mixtures with a large excess of alkaline phosphatase in a sealed capillary tube at 37°C. The mixtures were then chromatographed in system III and analyzed as described above.

RESULTS

Strain RTAE-1 spontaneously arose from an FU-treated culture of *M. thermoautotrophicum* Marburg and was isolated from a single colony. The strain grew thermophilically, autotrophically, produced methane, and was morphologically identical to strain Marburg. These properties are consistent with strain RTAE-1 being a strain of *M. thermoautotrophicum*. Resistance to FU was maintained after 20 passages in the absence of the compound. The resistant strain grew in the presence of 1 mg of FU per ml, more than 1,000 times the concentration which inhibited the wild type (50% inhibition at 1 μ g/ml) (31). When cultures of the wild-type strain were plated on solid medium with 100 μ g of FU per ml, the spontaneous mutation rate to FU resistance was determined to be $<1.6 \times 10^{-8}$. Both the wild-type strain and RTAE-1 grew in the presence of 5-fluorocytosine, but neither grew in the presence of 5-fluorodeoxyuridine (Table 1; 31). We also tested 5-azauracil and 6-azauracil as possible inhibitors of both strains. In a 24-h incubation with 600 μ g of 5-aza- or 6-azauracil per ml, the yield of wild-type cells was 12 and 22% of that of control cultures (without the compound) respectively, and the yield of strain RTAE-1 was 82 and 76% of that of the control cultures. Thus, the wild type was more susceptible to inhibition by these compounds than was strain RTAE-1 (data not shown).

To determine differences between the wild-type and FU-resistant strains which might be responsible for their phenotypes, growing cultures were incubated with ¹⁴C-labeled FU.

In the experiment for which results are shown in Fig. 1, the wild-type strain accumulated significantly more FU than did strain RTAE-1. Growth of the two cultures during the incubation was similar, although it was not quantitated. Based upon the initial cell densities present, at 4 h the wild-type culture had taken up 1.2 nmol/ 10^8 cells and strain RTAE-1 had taken up 0.07 nmol/ 10^8 cells, a difference of 17-fold, which was not accounted for by differences in growth.

To avoid the problem of toxicity of FU to the wild type, ^{14}C -labeled uracil was incubated with cells during growth. In the presence of uracil both strains grew at the same rate but clearly took up different amounts of radiolabel (Fig. 2). At 4 h the wild type had taken up 1.3 nmol/ 10^8 cells (the same amount per cell as in the FU experiment). The ratio of the amount of label in the wild-type cells to that in strain RTAE-1 was more than 5, a value which increased to 15 during the interval from 4 to 12 h. The uptake experiments were initiated by the addition of labeled compound to the cultures. Initial samples were taken as rapidly as possible, but the operations of flushing the syringe with anoxic gas, sampling, and filtration required 2 to 3 min. In this amount of time, cells of both strains bound significant amounts of label, which may have been a combination of adsorption to cells and rapid cellular import. If all the label was transported, the amount taken up by strain RTAE-1 at time zero (0.25 nmol/ 10^8 cells) corresponded to an internal uracil concentration of 3.6 mM, a value 75 times the external concentration. This calculation was based on assumptions that the labeled compound was not metabolized and that an individual cell weighed 10^{-12} g and consisted of 70% water by weight.

The metabolic fate of labeled uracil in strain Marburg was determined. Labeled cell pellets which had taken up 89% of

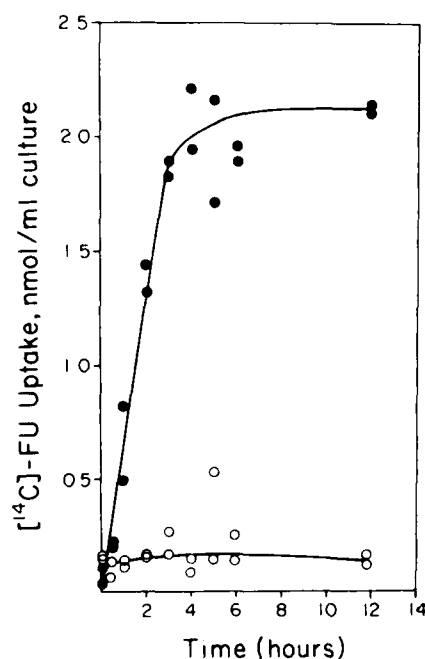


FIG. 1. Uptake of ^{14}C FU by *M. thermoautotrophicum* Marburg and RTAE-1. Growing cultures were treated with $41\ \mu\text{M}$ ^{14}C FU. 0.2-ml portions were filtered, and the amount of label retained was quantitated. The initial culture densities were 1.7×10^8 cells per ml for strain Marburg and 2.2×10^8 cells per ml for strain RTAE-1. Symbols: ●, strain Marburg; ○, strain RTAE-1.

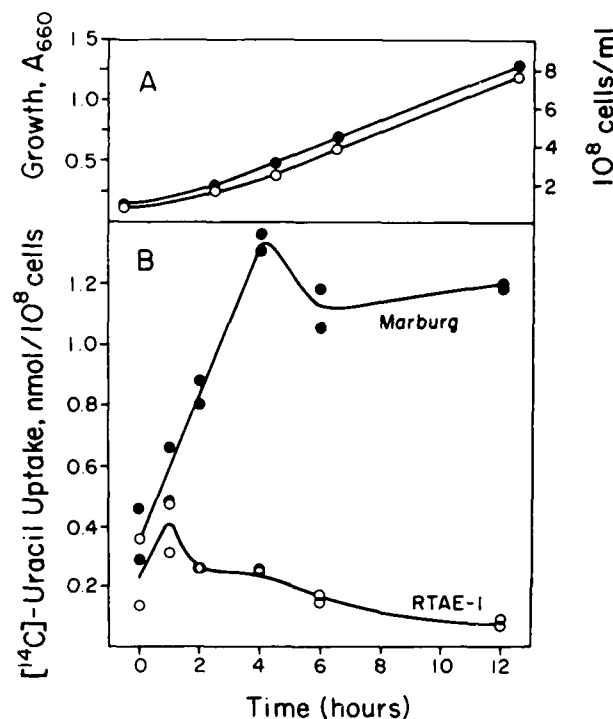


FIG. 2. Uptake of ^{14}C uracil by *M. thermoautotrophicum* Marburg and RTAE-1. (A) Growth. Cultures with ^{14}C uracil (identical to those in panel B except that ^{14}C uracil was omitted) were diluted for absorbance measurements. (B) Label incorporated by cultures treated with ^{14}C uracil ($48\ \mu\text{M}$). Label was added at time zero, and samples (0.2 ml) from cultures (20 ml) were removed anoxically, filtered, and quantitated. Symbols: ●, strain Marburg; ○, strain RTAE-1.

the uracil in the medium were subjected to TCA fractionation. We found that 29.7% of the radioactivity was in the cold TCA-soluble pool, 68.7% was in the hot TCA-soluble pool (mainly RNA), and 1.7% was in the hot TCA-insoluble pool (mainly DNA). These results showed that uracil was taken up and incorporated into macromolecules in *M. thermoautotrophicum*.

Since the FU-resistant strain RTAE-1 did not accumulate as much uracil or FU as did the wild type, a defect in metabolism may have been present. Therefore, we tested extracts for the presence of an enzymatic activity which could salvage uracil. Extracts of the wild-type strain contained significant levels of PRPP_i-dependent, UMP-forming activity (15% conversion of UMP in 1 h) (Table 2). Extracts of strain RTAE-1 had less than 10% of the activity of the wild type. When extracts of the two strains were mixed, there was no significant decrease in the activity of the wild-type strain, thus ruling out the presence of an inhibitor of the activity in the cell extracts of strain RTAE-1. The product of the reaction, UMP, was identified by comigration of radioactive material from reactions of wild-type extracts with authentic material in solvent systems I, II, and III. There was no significant conversion of UMP to other compounds under the assay conditions. Further evidence that UMP was the product was provided by alkaline phosphatase treatment of the products in reaction mixtures. Radioactivity comigrating with UMP was completely (99%) converted to a compound which comigrated with uridine after alkaline phosphatase treatment. When ribose-1-phosphate was sub-

TABLE 2. Uracil phosphoribosyltransferase activity in extracts of wild-type and FU-resistant *M. thermoautotrophicum* strains

Organism and assay condition ^a	Incubation time (h)	nmol of UMP/mg of protein
<i>M. thermoautotrophicum</i> Marburg		
Complete	1.0	6.3
- PRPP _i	1.0	0.1
Complete	6.0	12.6
- PRPP _i	6.0	2.0
- PRPP _i + ribose-1-phosphate	6.0	0.5
Complete + RTAE-1 extract	6.0	11.5 ^b
<i>M. thermoautotrophicum</i> RTAE-1 (complete)	1.0	0.5
	6.0	1.2
<i>E. coli</i> (complete)	1.0	1.1
	6.0	1.8

^a Complete assay mixtures included [¹⁴C]uracil, PRPP_i, MgCl₂, buffer, and protein (3 to 5 mg/ml) in 100 µl. The methanogen extracts were incubated at 55°C, and the *E. coli* extracts were incubated at 37°C. After incubation, portions were spotted on cellulose thin-layer plates and chromatographed in solvent system I, and the radioactivity in segments of the lane was quantitated. The amount of UMP synthesized was corrected for that which appeared in incubations without enzyme.

^b Calculated on the basis of strain Marburg protein present; an equal portion of strain RTAE-1 extract was added.

stituted for PRPP_i in the extract from strain Marburg, there was no evidence for formation of the nucleoside uridine.

The enzyme activity in the wild type was oxygen stable but decreased upon repeated thawing and freezing of extracts. The results shown in Table 2 were from a typical experiment. The amount of UMP synthesized in 1 h by wild-type extracts varied from 5 to 10 nmol/mg, but strain RTAE-1 extracts never formed more than 0.5 nmol/mg. There was no significant difference in activity in incubations done at 55 and 63°C and no variability which could be ascribed to differences in extracts from independently grown cells. However, the activities were not computed as rates because the amount of UMP synthesized was not linearly proportional to time or to the amount of protein. The labile nature of the substrate PRPP_i probably contributed to this observation. At the elevated temperatures required for the thermophilic organism, PRPP_i appeared to be limiting. In some experiments in which additional PRPP_i was added to assay mixtures at 15-min intervals, we obtained UMP synthesis levels 2 to 5 times higher than those shown in Table 2 and as much as 63% substrate conversion in 3 h. *E. coli*, used as a control organism, had low levels of enzyme activity (Table 2). We did not attempt to optimize assay or growth conditions with *M. thermoautotrophicum* or *E. coli*.

DISCUSSION

FU inhibits the growth of *M. thermoautotrophicum*. We have previously shown that this drug does not act directly on methanogenesis (31). Since uracil but not cytosine, thymine, uridine, or thymidine spared the cells from inhibition, there is specificity in transport or metabolism of FU (31). Since labeled uracil was taken up and incorporated into RNA and DNA, the organism must have a mechanism to salvage uracil. The data in Table 2 show that part of the uracil activation system in the wild type was a PRPP_i-dependent, UMP-forming enzyme activity. The amounts of activity detected were significantly less than those in a eubacterium which requires preformed pyrimidines (20), in *E. coli* or

Salmonella typhimurium strains induced for the enzyme (3, 5, 27), or in the yeast *Saccharomyces cerevisiae* (24). However, they were the same as those found in cultured plant cells which do not require pyrimidines (14). *M. thermoautotrophicum* is an autotroph and might not be expected to need much pyrimidine salvage activity.

The mechanisms by which FU and the azauracil compounds inhibit the growth of *M. thermoautotrophicum* are not known. In eubacteria and eucaryotes, nucleotide metabolites of these compounds are the actual inhibitors. For example, FU is metabolized to 5-fluorodeoxyuridylate, a potent inhibitor of thymidylate synthesis. Without thymidylate, DNA synthesis cannot occur (12). FU is also metabolized to 5-fluorouridine triphosphate and is incorporated into RNA, and FU-substituted RNAs cannot function as efficiently as normal forms (19). We have observed that thymine or thymidine do not spare the cells from inhibition by FU (31). If either of these is activated to the nucleotide form, DNA synthesis ought to be able to continue despite the presence of FU. Thus, it is possible that FU metabolites incorporated into RNA disrupt its function in the methanogen. The azauracil compounds are activated to nucleotide derivatives which inhibit de novo pyrimidine biosynthesis (28). We have not studied the reversal of inhibition by the azauracil compounds.

The FU-resistant strain RTAE-1 grew at the normal rate at levels of FU 10³ times those which inhibited growth of the wild type. The strain was also less susceptible to inhibition of growth by 5-azauracil and 6-azauracil than was the wild-type strain. When strain RTAE-1 was grown in the presence of radiolabeled uracil or FU, the cells failed to accumulate label. The amounts incorporated at the first sampling time appeared to be more than could be accounted for by nonspecific binding, which ought to increase as the number of cells increases. These data might be explained by altered permeability to the compounds in strain RTAE-1. Resistance to pyrimidine analogs in certain mutants of the fungi *Neurospora crassa* and *S. cerevisiae* is accounted for by decreased permeability (6, 9). However, since extracts of strain RTAE-1 contained less than 10% as much uracil phosphoribosyltransferase activity as did the wild type, it appeared that the uracil analogs were not activated. Both FU resistance in the eubacteria (Upp phenotype) and 6-azauracil resistance in plant tissue culture cells result from the loss of uracil phosphoribosyltransferase (3, 5, 14, 25, 26). Definitive proof that the resistance of the strain was not due to a combination of lowered permeability and loss of uracil phosphoribosyltransferase activity will require transport studies. However, the decrease in the levels of the uracil salvage enzyme in the FU-resistant strain RTAE-1 may be sufficient to account for its resistant phenotype.

Mutation to drug resistance may result from changes in the genes for (i) transport of the compound, (ii) the site of action, or (iii) activation of the compound to the inhibitory form. Metabolic inactivation by a degradative enzyme activity will not be considered here. For methanogens, alternative i holds for mutants with resistance to neomycin or virginiamycin (*Methanococcus voltae*) or bromoethanesulfate (*M. voltae* and *Methanosarcina barkeri*). In these instances, the evidence suggests that the resistant cell is less permeable to the compound than is the wild type (11, 29, 30). A similar explanation has been proposed for other resistances (11, 17).

There are two examples of mechanism ii, altered site of action, in methanogens. The ribosome of an anisomycin-resistant *Methanobacterium formicicum* strain is not inhibited by the compound; the cells are cross-resistant to several

other ribosome inhibitors (13). The isoleucyl-tRNA synthetase in a pseudomonic acid-resistant *M. thermoautotrophicum* strain is not inhibited by the compound, unlike the wild-type enzyme (18).

FU-resistant strain RTAE-1 appears to be an example of alternative iii, resistance to an antimetabolite because of a deficient activation process. Strain RTAE-1 was deficient in uracil phosphoribosyltransferase and refractory to inhibition by FU and azauracils. It is likely that the type of pyrimidine (and probably purine) analog resistance, "namely, failure to form fraudulent ribonucleotide derivatives from the corresponding base" (5), found in strain RTAE-1 will be common in archaeobacteria as it is in eubacteria and eucaryotes. Purine and pyrimidine analog resistances have been reported for strains of *M. voltae* (T. L. Bowen and W. B. Whitman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, 147, p. 172), and we have isolated 6-mercaptopurine- and 8-azaguanine-resistant *M. thermoautotrophicum* strains (V. Worrell, D. McCarthy, and D. P. Nagle, Jr., unpublished results). Work is in progress to define the site of action of FU and to develop a genetic exchange system based on our resistant strains.

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